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CHAPTER TWO

Expression of an 8*R*-Lipoxygenase From the Coral *Plexaura homomalla*

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Contents

Abstract

Methods are presented for the use of the coral 8*R*-lipoxygenase from the Caribbean sea whip coral *Plexaura homomalla* as a model enzyme for structural studies of animal lipoxygenases. The 8*R*-lipoxygenase is remarkably stable and can be stored at 4°C for 3 months with virtually no loss of activity. In addition, an engineered "pseudo wild-type" enzyme is soluble in the absence of detergents, which helps facilitate the preparation of enzyme:substrate complexes.

1. INTRODUCTION

The discovery of prostaglandins in *Plexaura homomalla* in 1969 (Weinheimer & Spraggins, 1969) eventually led to the identification of cyclooxygenase (COX) and lipoxygenase (LOX) biosynthetic pathways in corals. While the functions of the oxylipins these enzymes generate

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remain undefined, *P. homomalla* 8*R*-LOX has proven to be a remarkably stable homologue of human 5-LOX, with which it shares ~40% sequence identity. This is the same level of sequence identity observed between 5-LOX and its closest intraspecies orthologue 15-LOX-2. Human 5-LOX is the only clinically validated drug target of the LOX family, but the enzyme's short half-life has made biochemical characterization difficult (Carter et al., 1991; Percival, Denis, Riendeau, & Gresser, 1992; Wenzel et al., 2007). The extraordinary stability of the coral enzyme makes it a highly tractable system for structural studies, as protein instability is a frequent roadblock to structural studies (Deller, Kong, & Rupp, 2016), and 8*R*-LOX structures have revealed key mechanistic details of the LOX oxygenation reaction. Although 8*R*-LOX was originally identified as the C-terminal region of a naturally occurring fusion protein (Gilbert et al., 2008; Koljak, Boutaud, Shieh, Samel, & Brash, 1997), we will focus on the isolated LOX domain in this report (Boutaud & Brash, 1999; Brash, Boeglin, Chang, & Shieh, 1996; Oldham, Brash, & Newcomer, 2005).

LOXs are nonheme iron enzymes that catalyze the regio- and stereospecific dioxygenation of polyunsaturated fatty acids at a *cis*, *cis*-1,4-pentadiene (Boutaud & Brash, 1999), often to generate a potent lipid-signaling mediator. The enzymes have been found in animals, plants, bacteria, and fungi, with certain species harboring a catalytic manganese instead of iron (Andreou & Feussner, 2009; Boutaud & Brash, 1999; Hamberg, Su, & Oliw, 1998; Kuhn & Thiele, 1999). The common substrate for animal LOXs is arachidonic acid (AA), an ω6 20-carbon fatty acid with four double bonds. There are 3 pentadienes in AA with the potential for 12 unique oxidation products that can be produced by a single LOX reaction (Fig. 1A). The overall tertiary structure of plant (Boyington, Gaffney, & Amzel, 1993) and animal (Gillmor, Villasenor, Fletterick, Sigal, & Browner, 1997) LOXs shares a common fold. These LOXs contain an N-terminal β-barrel domain of ~15 kDa, which may harbor Ca^{2+}-binding residues and membrane-insertion loops that target the enzyme to the membrane periphery (Fig. 1B). The much larger (~4 ×) C-terminal domain of LOX positions the catalytic iron at the base of a U-shaped cavity (Fig. 1C). The Fe^{2+} is chelated by invariant histidines and the main chain carboxy terminus (Minor et al., 1996; Newcomer & Brash, 2015; Skrzypczak-Jankun, Bross, Carroll, Dunham, & Funk, 2001; Xu, Mueser, Marnett, & Funk, 2012).

Our initial work with the 8*R*-LOX domain of the *P. homomalla* fusion protein led to a 3.2 Å resolution structure (Oldham et al., 2005).

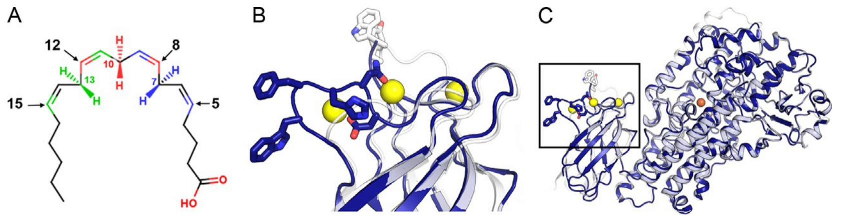

Fig. 1 Lipoxygenase reaction and structure. (A) Arachidonic acid has three pentadienes with hydrogens on carbon 7, 10, and 13 (*smaller font* and *colored blue*, *red*, and *green*, respectively, or *different shades of gray*) that are possible sites of attack by LOX enzymes. Notice in the online version of the figure that carbons are colored with respect to the hydrogen that needs to be abstracted for oxygenation to occur at that carbon. For example, hydrogen abstraction on carbon 10 (*red*) will result in oxygen addition at either carbon 8 or 12 (both *red*), depending on the particular enzyme. (B) 8*R*-LOX WT (PDB code 2fnq) and 8*R*-LOX psWT (PDB code 3fg1) are superimposed for comparison. Membrane-insertion loops and Ca^{2+}-binding residues have been deleted from the latter. Detailed view of N-terminal domain (*framed box* in panel C) with 8*R*-LOX WT shown in *blue* or *dark gray* and 8*R*-LOX psWT shown in *white*. *Spheres* are bound Ca^{2+} as seen in the 8*R*-LOX WT structure. Residues (*blue/dark gray*) that are highlighted are either Ca^{2+}-binding residues 43–45 (His-Asn-Asp) or membrane-insertion residues 41–42 (Trp-Phe). Residues shown in *white* are membrane-binding residues Tyr 77 and Trp 78. (C) Cartoon rendering of 8*R*-LOX WT shown in *blue* or *dark gray* and 8*R*-LOX psWT shown in *white*. The catalytic domain is mainly α-helical and harbors the catalytic iron (*orange/gray sphere*).

Examination of crystal packing led us to suggest that a membrane-insertion loop interfered with packing of the protein molecules in the crystal lattice. We reasoned that removal of an extended hydrophobic loop, which penetrated a neighboring molecule, might facilitate a tightly packed lattice that could provide us with higher resolution X-ray data (Fig. 1B). Deletion of a membrane-insertion loop (8*R*-LOX Δ41-45GS) from the PLAT (polycystin-1, LOX, Alpha-Toxin) domain of 8*R*-LOX by mutagenesis yielded an enzyme with increased solubility (Fig. 2). The construct was given the moniker "pseudo-wild type (psWT)" because it retains the in vitro enzymatic activity of its progenitor, despite the lack of both a membrane-binding loop and Ca^{2+}-binding amino acids necessary for membrane targeting (Neau, Gilbert, Bartlett, Dassey, & Newcomer, 2007). As hoped, the mutant provided crystals that diffract to 1.9 Å resolution and amenable to the manipulation required for an anaerobic enzyme:substrate complex (Neau et al., 2014, 2009). The latter structure revealed the AA substrate positioned in the U-shaped active site in a pose consistent with the regio- and stereospecificity of 8*R*-LOX, and hence provided a robust model

Fig. 2 Sequence alignment of four human lipoxygenases with the coral 8*R*-LOX and its deletion mutant psWT. The PDB codes are included if a sequence from a solved protein structure was used. The *box frames* the area of the protein sequence where Ca^{2+}-binding residues and membrane-insertion residues were mutated. The * represents amino acids that bind Ca^{2+} in the N-terminal domain. The • represents amino acids known to peripherally bind the membrane. The amino acid sequence WFHND of 8*R*-LOX was mutated to GS to generate 8*R*-LOX psWT. The amino acid sequence PFYND of 5-LOX was mutated to GS to generate a soluble 5-LOX known as Stable-5-LOX.

for understanding LOX product specificity in this superfamily (Newcomer & Brash, 2015). The protein engineering strategy of removing a LOX membrane-insertion loop has been applied to additional LOX (Mittal et al., 2017) and yielded a protein amenable to crystallization (Gilbert et al., 2011).

2. PREPARATION OF HIGHLY PURIFIED 8*R*-LOX

Having homogenous and monodisperse protein is generally an important prerequisite for obtaining diffraction-quality crystals suitable for high-resolution structure determination. For the case of 8*R*-LOX, an ample amount of protein can be expressed in *Escherichia coli* with low temperature incubation of the cultures in the autoinduction media first described by Studier (2005).

2.1 Transformation of 8*R*-LOX psWT Into Overexpression Host

The pET-3a plasmid harboring 8*R*-LOX psWT is transformed into BL21 (DE3) cells using the heat-shock protocol.

2.1.1 Equipment

- 42°C water bath for heat-shock transformation
- 37°C benchtop shaking incubator
- 37°C incubator for overnight incubation of agar plates

2.1.2 Buffers and Reagents

- *E. coli* BL21 (DE3) cells (Invitrogen)

- SOC media—2% tryptone, 0.5% yeast extract, 10 m*M* NaCl, 2.5 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* $MgSO_4$, and 20 m*M* glucose (Invitrogen)
- Luria–Bertani (LB) broth with 10 g peptone, 5 g yeast extract, and 5 g NaCl (Research Products International)
- Carbenicillin (100 μg/mL final concentration) (P212121)

2.1.3 Procedure

1. 10 μL of *E. coli* BL21 (DE3) cells are thawed on ice. 50 ng of plasmid DNA is added to thawed cells and tube is flicked 4–5 times to mix the cells.
2. The mixture is incubated on ice for 5 min.
3. The mixture is placed in a 42°C bath for 45 s and returned back to ice for 2 min.
4. 200 μL of prewarmed SOC media is added, and the mixture is transferred to a 14-mL round bottom falcon tube.
5. The tube is placed at 37°C for 45 min in incubator and is vigorously rotated.
6. The mixture is plated on LB plate with carbenicillin and incubated overnight at 37°C.

2.1.4 Notes

1. Transformation efficiency is reduced with multiple freeze/thaw cycles of the competent cells.
2. We routinely only use 10 μL of competent cells, which is one-fifth of the volume aliquoted by the supplier. The other portion of competent cells is transferred to sterile 1.5-mL microcentrifuge tubes in aliquots of 10 μL and placed in −80°C.
3. We do not store glycerol stocks of transformed bacteria as they do not necessarily provide the generous yields that freshly transformed bacteria do. We invariably start each preparation with freshly transformed cells.

2.2 Expression of 8*R*-LOX

2.2.1 Equipment

- Large, high-capacity floor model orbital shaker with refrigeration capabilities (Infors HT, Multitron model)
- Standard SDS-PAGE equipment, 10% SDS-PAGE gels

2.2.2 Buffers and Reagents

- Autoinducing media ZYM-5052 is a media for growing high-density shake-flask cultures that upon saturation will autoinduce protein expression (Studier, 2005). ZYM-5052 contains:
  - **i.** 1% N-Z-amine AS, 0.5% yeast extract, abbreviated *ZY* and is autoclaved separately
  - **ii.** 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, abbreviated $50\times M$ and is autoclaved separately at $50\times$ concentration
  - **iii.** 2 mM $MgSO_4$, made at a concentration of 1.0 M and autoclaved separately
  - **iv.** 0.5% glycerol, 0.05% glucose, 0.2% α-lactose, abbreviated as *50× 5052*, made at 50× concentration, and sterile filtered
- Optional: IPTG (isopropyl β-D-1-thiogalactopyranoside) used at 500 μM final concentration (Research Products International)

2.2.3 Procedure

1. A single colony from the plate is used to inoculate (2×) 25 mL LB in 125-mL Erlenmeyer flask with carbenicillin for selection.
2. Shake flasks are grown overnight in the incubator at 220 rpm, 37°C.
3. (8×) 480 mL of *ZY* media is prepared in a nonbaffled 2-L Erlenmeyer flask the day before inoculation along with stocks of 1.0 M $MgSO_4$, $50\times M$, and 50×5052. (Pro hint: LOX expression is very sensitive to aeration of the media. Flask size, baffles vs nonbaffles, and temperature along with rpm should be followed carefully according to protocol).
4. 2.0 mL of the overnight culture is added to each flask along with 10 mL of $50\times M$ and 50×5052, and 1 mL of 1.0 M $MgSO_4$.
5. Cultures are grown at 37°C, 220 rpm for 4 h.
6. The temperature is dropped to 20°C and grown overnight. On the next morning, the optical density of the cultures is monitored by absorbance at 600 nm. It is key to make sure that the bacterial growth is past log phase and has entered the stationary phase otherwise autoinduction does not occur. Upon saturation of culture, lactose is transported inside the bacterial cell, and β-galactosidase converts the lactose to allolactose. Allolactose induces expression of the protein of interest by binding the lac repressor, which now allows T7 RNA polymerase to bind the lac promoter and produce the mRNA transcripts for 8*R*-LOX psWT.

7. The cells usually reach saturation that afternoon and are harvested and centrifuged at $5000 \times g$ for 10 min. Pellets are frozen at −80°C until further use.

2.2.4 Notes

1. IPTG may be added 4 h before harvest if uncertain about having reached a stationary phase and therefore having undergone autoinduction overexpression of 8*R*-LOX psWT.
2. Protein expression can be checked by taking a 1-mL sample of growth at harvest. The sample is spun down at 13,000 rpm in microcentrifuge. A volume of 300 μL of Bugbuster® is added and then incubated at 4°C while nutating for 1 h. Soluble and insoluble material is separated by centrifugation at 13,000 rpm and samples are run on SDS-PAGE gel. 8R-LOX psWT migrates ~75 kDa and should appear in the soluble fraction.

2.3 Purification of 8*R*-LOX

This three-step purification can be completed in 2 days and yields highly purified enzyme (~10 mg enzyme per 1 L of expression media) that is amenable to high-resolution structural studies (Fig. 3).

2.3.1 Equipment

- Branson Sonifier 250 (VWR Scientific)
- FRENCH® Pressure cell press (SLM Instruments, Inc. of Milton Roy company)
- Nalgene syringe filter, 25 mm cellulose acetate membrane, 0.8 μm (ThermoFisher Scientific)
- DynaLoop 90, store in 20% ethanol (Bio-Rad)
- AKTA FPLC (GE Healthcare Life Sciences)
- HisTrap HP 5 mL column (GE Healthcare Life Sciences)
- Dialysis tubing with average flat width 25 mm, 14 kDa cutoff (Sigma). Tubing is washed under running water for 2 min before use. Residual glycerin and sulfur compounds from dialysis tubing do not appear to affect purification of the 8*R*-LOX psWT protein.
- Mono-Q (10/100 GL) anion exchange (GE Healthcare Life Sciences)
- Spin-X® UF 20 mL Concentrator 30,000 molecular weight cutoff (Corning)
- Nanodrop™ 2000 Spectrophotometer (ThermoFisher Scientific) Molecular weight of protein is 79,000 Da and theoretical molecular

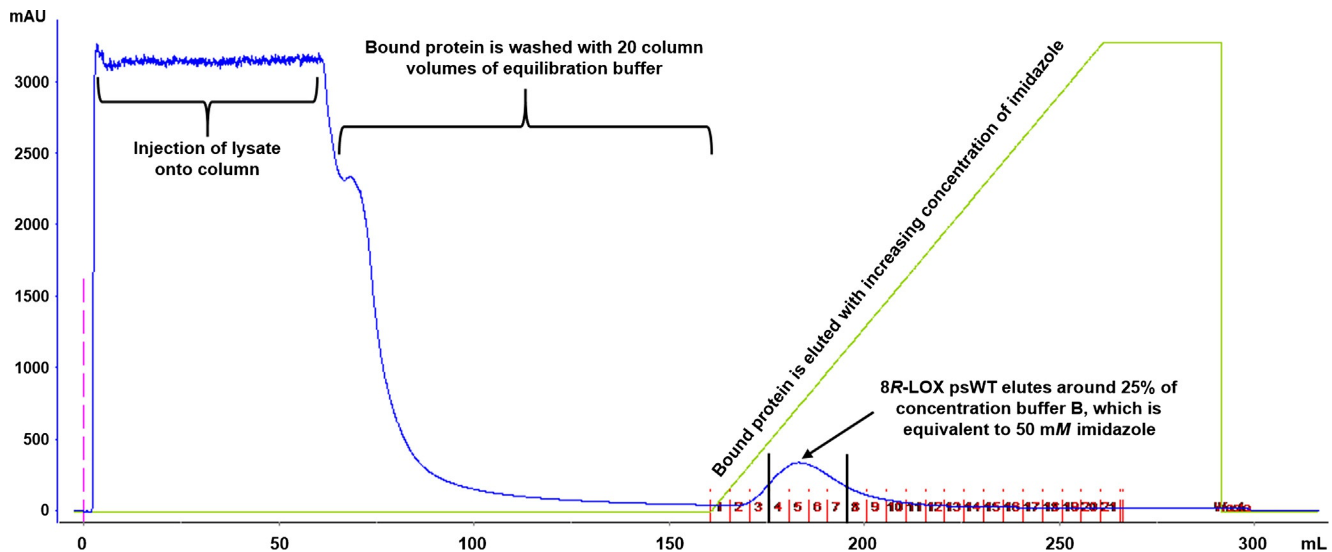

Fig. 3 Purification of 8*R*-LOX psWT by immobilized metal-affinity chromatography. The *y*-axis is the milli-absorbance at 280 nm, and *x*-axis the elution volume. Cell lysate (80 mL) was loaded onto a HisTrap HP 5 mL column followed by 100 mL of wash with equilibration buffer. Bound 8*R*-LOX psWT is eluted by a gradient of increasing concentration of buffer B (200 m*M* imidazole). Fractions 4 through 7 were collected and used for further purification.

extinction coefficient that is used is 137,000 for measuring protein concentration.

- Hiload 16/600 Superdex 200 10/300 PG (GE Healthcare Life Sciences)
- Vial Clamp™ curved (Hampton Research)
- Long-handled tweezers with serrated tip and furrowed handle (Grainger)
- Low form shallow dewar with hemispherical bottom (Pope Scientific Inc.) (see Fig. 4)

2.3.2 Buffers and Reagents

- Bugbuster®, bacterial chemical lysis reagent with detergent (Novagen)
- DNase I from bovine pancreas (Sigma)
- Leupeptin (5 μg/mL final concentration), pepstatin (10 μ*M* final concentration, Sigma), and PMSF (phenylmethylsulfonyl fluoride, Sigma)
- Immobilized affinity chromatography (IMAC) buffer A: 20 m*M* Tris pH 8.0/500 m*M* NaCl/20 m*M* imidazole pH 8.0
- IMAC buffer B: 20 m*M* Tris pH 8.0/500 m*M* NaCl/200 m*M* imidazole pH 8.0
- Anion-exchange buffer A: 20 m*M* Tris pH 8.0
- Anion-exchange buffer B: 20 m*M* Tris pH 8.0 and 500 m*M* NaCl
- Size exclusion chromatography (SEC) buffer: 20 m*M* Tris pH 8.0 and 150 m*M* NaCl

Fig. 4 Cryocooling of purified 8*R*-LOX psWT. The low form shallow dewar with hemispherical bottom is shown without liquid N_2. The curved vial clamp™ and tweezers have been placed on top of the dewar. Inset in *bottom left corner* shows protein that has been cryocooled in the liquid N_2, collected by long-handled tweezers, and placed into cryocooled microcentrifuge tube that is held by the curved vial clamp™. Protein beads are not clearly discerned due to fogging of cryocooled tube.

2.3.3 Procedure

1. Cell pellets containing expressed 8*R*-LOX psWT are weighed out in a 250-mL plastic beaker. Typical growth from 4 L of media results in ~45 g of cell pellet.
2. Bugbuster® is added at customized ratio of 2.0 mL of Bugbuster® per 1.0 g of cell pellet. Manufacturer recommends 5.0 mL of Bugbuster® per 1.0 g of cell pellet.
3. DNase I (100 Kunitz) is added along with PMSF (~2 mg of crystalline material), pepstatin, and leupeptin. The solution is homogenized on a stir plate for 10 min in an ice-bath.
4. Mixture can either be:
   - **a.** Sonicated (3 ×) for 2 min, 50% duty, and output control of 8 out of 10 on Branson Sonifier 250. Between cycles of sonication, mixture is cooled on ice for 5 min.
   - **b.** French pressed with pressure >16,000 psi on FRENCH® Pressure cell press. The French pressure system that is utilized allows ~40 mL of mixture to be pressed per pass. Typical output time is 5 min per 40 mL. Once the mixture is passed through the press, the solution is immediately placed in an ice-bath. Note: French press lysis yields larger amounts of protein due to better lysis of cells and less loss due to denaturation.
5. Mixture is centrifuged at 40,000 × *g* for 45 min at 4°C to clarify lysate and to pellet insoluble portion.
6. Supernatant is filtered by 0.8-μm syringe filter and loaded onto superloop (DynaLoop 90).
7. HisTrap HP 5 mL column is equilibrated with 20 m*M* Tris pH 8.0/500 m*M* NaCl/20 m*M* imidazole pH 8.0 on FPLC until UV and conductivity are stable baselines (usually 5 column volumes, make sure to pH both Tris and imidazole).
8. Supernatant from superloop is loaded onto column at 2 mL/min.
9. Column with bound protein is washed with 100 mL of equilibration buffer.
10. Protein is eluted using a linear gradient of 100% B over 20 column volumes with 5 mL fractions. Buffer B is similar to equilibration buffer but with 200 m*M* imidazole pH 8.0.
11. Fractions that contain protein as monitored by absorbance at 280 nm are collected from the middle of the Gaussian peak. Shoulder fractions lower than one-third of the peak height are discarded.

12. Peak fractions are pooled and placed in dialysis tubing and dialyzed overnight in 2 L of 20 m*M* Tris pH 8.0 in cold room.
13. The next day, the Mono-Q anion-exchange column is equilibrated with 20 m*M* Tris pH 8.0 on the FPLC until the UV and conductivity signals have stable baselines.
14. Protein that was dialyzed overnight is syringe filtered and loaded onto cleaned superloop.
15. Protein is injected onto Mono-Q from the superloop at 2 mL/min and the column is washed with 40 mL (5 column volumes) of equilibration buffer.
16. Protein is eluted with linear gradient from 0% to 100% B over 10 column volumes with 5 mL fractions. Buffer B contains 20 m*M* Tris pH 8.0 and 500 m*M* NaCl.
17. Once again fractions that contain protein as indicated by A_{280} are collected from the middle of the Gaussian peak using a similar shoulder cutoff.
18. Peak fractions are pooled and concentrated in Spin-X® UF 20 mL Concentrator with 30,000 molecular weight cutoff. Protein is concentrated to ~2 mL and 20 mg/mL as measured on a Nanodrop™.
19. Protein is loaded onto 2.0 mL loop and injected onto Hiload 16/600 Superdex 200 10/300 PG equilibrated in 20 m*M* Tris pH 8.0/150 m*M* NaCl.
20. Monomeric protein elutes ~80 mL, and 1 mL peak fractions are pooled and concentrated in Spin-X® UF 20 mL Concentrator with 30,000 molecular weight cutoff until protein reaches 10 mg/mL.
21. Protein purity is accessed by a 10% SDS-PAGE gel run at 150 V for 75 min.
22. Purified protein is slowly dripped from a transfer pipette into liquid N_2 in a shallow dewar. The protein beads are typically ~25 μL. The protein beads are collected into a cryocooled microcentrifuge tube held with vial clamps, and subsequently placed in −80°C freezer.

2.3.4 Notes

1. For cell lysis, our lab usually both sonicates and then uses the French press. Sonication is used to make the sample more homogeneous, which makes using the French press easier. Just make sure not to overheat the lysate solution.

2. The expression yield of 8*R*-LOX psWT is robust so do not be afraid to cutoff shoulders of protein peaks. We see more reproducible crystal growth conditions for 8*R*-LOX psWT when we keep only the peaks from the chromatography runs.
3. When freezing the purified 8*R*-LOX psWT into liquid N_2, drip the protein just a few drops at a time. This allows for independent freezing of the protein drops instead of protein drops coming together to make larger aliquots.
4. After placing protein drops into cryocooled microcentrifuge tube, make sure that there is no residual liquid N_2 in the tube before closing. Failure to boil all liquid N_2 from the microcentrifuge tubes can result in the lid blowing off along with your protein beads spilling onto the floor. (Hint: I hold microcentrifuge tube in fingers for about 30 s before closing lid. This is about the time it takes me to walk to the −80°C freezer.)

3. STRUCTURAL STUDIES OF 8*R*-LOX

The molecular weight of 8*R*-LOX at 79 kDa makes crystallography the ideal tool for structural studies, with the enzyme being too large for NMR and too small for Cryo-EM. Crystallization of 8*R*-LOX or 8*R*-LOX psWT has only been successful in conditions that include Ca^{2+}, a cofactor for the membrane-insertion loops on the N-terminal domain (Neau et al., 2014, 2009, 2007; Oldham et al., 2005). To get an enzyme:substrate complex trapped, the crystals must be grown in an anaerobic environment. LOXs insert molecular O_2 on the fatty acid, so growing 8*R*-LOX psWT crystals in an anaerobic environment is the first step to obtaining an enzyme:substrate complex. The second step is soaking these anaerobic crystals with AA in a glove box and cryocooling in the anaerobic environment before X-ray data collection.

3.1 Anaerobic Crystallization of 8*R*-LOX

3.1.1 Equipment

- Vinyl anaerobic chamber (Coy Laboratory Products)
- Sitting drop crystallization plate, such as the Cryschem Plate (Hampton Research)
- Crystal Clear Sealing Tape or Crystal Clear Sealing Film (Hampton Research)
- Mounted cryoloop for crystal harvesting (Hampton Research)

3.1.2 Buffers and Reagents

- 10 mg/mL 8*R*-LOX psWT in 150 m*M* NaCl, 20 m*M* Tris, pH 8.0
- 25% (w/v) PEG-8000 (FLUKA brand)
- 50% (v/v) Glycerol
- 2 *M* $CaCl_2$
- 1 *M* Imidazole acetate, pH 8.0

3.1.3 Procedure (Carried Out Inside the Anaerobic Chamber)

1. Crystallization reagents and buffers should be degassed before bringing them into the anaerobic chamber. Solutions should be transferred to the anaerobic chamber immediately after degassing to keep them from absorbing oxygen. Two alternative methods of degassing are presented below.
   a. Solutions can be degassed by placing them into a round bottom flask and pulling a slight vacuum on the flask while stirring or sonicating the solution and then refilling the flask with an inert gas, such as nitrogen or argon. This should be done 3–5 times, pulling the vacuum for about 5 min each time before refilling with the inert gas.
   b. Solutions can also be degassed by sealing them in a flask and bubbling an inert gas through the solution while sonicating for 30 min to an hour.
2. In the well of the crystallization tray, mix the following amounts of the crystallization reagents:
   - 200–320 μL 25% PEG-8000 (200 μL in first row, increase by 40 μL each row after)
   - 100 μL 50% Glycerol
   - 100 μL 2 *M* $CaCl_2$
   - 100 μL Imidazole acetate, pH 8.0
   - Bring volume of well to 1000 μL by addition of deionized water

   This creates well solutions of 5%–8% PEG-8000, 5% glycerol, 0.2 *M* $CaCl_2$, and 0.1 *M* imidazole acetate, pH 8.0
3. Place 4 μL of 10 mg/mL 8R-LOX psWT on all of the pedestals of the crystallization plates, then immediately pipette 4 μL of well solution into the protein drop.
4. Seal the top of the crystallization plate using either clear sealing tape or clear sealing film.
5. Incubate the trays at room temperature. Crystals should appear in 1–2 days. Crystals typically grow with a rod-shaped, rhombic crystal habit. Occasionally the crystals will appear more blocky than rod-like.

6. Cryosolution for cooling of crystals is prepared by mixing 500 μL of 50% glycerol, 400 μL of 25% PEG-8000, 10 μL of 2 *M* $CaCl_2$, and 100 μL of imidazole acetate, pH 8.0 (final solution is approximately 25% glycerol, 10% PEG-8000, 0.02 *M* $CaCl_2$, 0.1 *M* imidazole acetate, pH 8.0).
7. Crystals are prepped for data collection by using a sharp blade to cut away the sealing film over a crystallization drop then looping a crystal from the crystallization drop with a mounted cryoloop and transferring it into a small drop (5–10 μL) of cryosolution. The crystal can then be immediately looped again and plunged into liquid N_2 to vitrify the crystal.

3.1.4 Notes

1. Aerobic crystals of 8*R*-LOX psWT can be grown using the same procedure as above, simply set up trays outside of the anaerobic chamber. In this case, solutions do not need to be degassed and crystals can be grown in hanging drop crystals plates as an alternative to the sitting drop plates.
2. It is not feasible to degas the protein solution. Simply transfer the amount of protein needed for crystallization into the anaerobic chamber in an open Eppendorf tube.
3. Liquid N_2 can be brought into the anaerobic chamber in a bowl dewar. When taking the liquid N_2 through the antechamber it has a tendency to boil violently during the first purge cycle of the antechamber. If this boiling causes too much liquid N_2 to spill in the antechamber, it will volatilize quickly enough to overpressure the antechamber, potentially contaminating the anaerobic chamber. To minimize this risk, pull vacuum slowly during the first purge cycle if the vacuum is manually controlled. Liquid N_2 tends to make the oxygen level rise in the anaerobic chamber as it boils off. As long as oxygen stays below ~50 ppm, the system should still be considered anaerobic.

3.2 Preparation of 8R-LOX:AA Binary Complexes

3.2.1 Equipment

- Small plastic petri dishes (1.5–2 in. in diameter)
- Syringe of vacuum grease. Hint: use a normal plastic syringe (maybe 5 or 10 cc) filled with vacuum grease and place a P200 pipette on the tip of the syringe for fine control as an applicator.

3.2.2 Materials

- Anaerobic 8*R*-LOX psWT crystals
- Arachidonic acid

- Anaerobic 8*R*-LOX psWT cryosolution: 25% glycerol, 10% PEG-8000, 0.02 *M* $CaCl_2$, 0.1 *M* imidazole acetate, pH 8.0
- DMSO

3.2.3 Procedure (Carried Out Inside the Anaerobic Chamber)

1. Dilute AA to 100 mg/mL using DMSO.
2. Dilute the AA further to 1 mg/mL by adding 1 μL of the 100 mg/mL AA solution to 99 μL of the 8*R*-LOX psWT cryosolution.
3. Apply a bead of vacuum grease to the inside edge of the lid of the petri dish.
4. Place 1.0 mL of 8*R*-LOX psWT cryosolution into the bottom of the petri dish.
5. Put a 5.0 μL drop of the 1 mg/mL AA solution on the inside surface of the lid of the petri dish.
6. Using a mounted cryoloop, transfer an anaerobic 8*R*-LOX psWT crystal into the drop of AA solution.
7. Invert the lid and seal it onto the bottom of the petri dish, so that the drop containing the crystal is suspended over the 1.0 mL of cryosolution. The 1.0 mL of cryosolution prevents the soaking drop from drying out.
8. Allow the crystal to soak overnight (~16 h).
9. Loop the crystal from the soaking drop and immediately plunge into liquid nitrogen.

3.2.4 Notes

1. Cocrystallization of 8*R*-LOX psWT with AA has not been successful. A relatively high concentration of $CaCl_2$ is required to crystallize 8*R*-LOX, and AA is insoluble when at this concentration of $CaCl_2$.
2. Trinary complexes of 8*R*-LOX, AA, and Xenon (an oxygen mimic) should be achievable by preparing the binary complex as above and then transferring the soaked crystals into a Xenon pressurization chamber.

4. SUMMARY AND CONCLUSIONS

The 8*R*-LOX from *P. homomalla* is an excellent model enzyme for animal LOXs due to its robust overexpression and inherent protein stability. Our lab's undergraduate research students routinely execute the expression and purification of 8*R*-LOX psWT. These aforementioned methods are used as a training manual for new students, and these methods form the foundation for developing expression and purification protocols for additional LOX enzymes. Our current understanding of animal LOXs has been greatly expanded with the multiple crystal structures of 8*R*-LOX, 8*R*-LOX psWT, and 8*R*-LOX psWT with substrate.

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